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Proceeding Paper

Quantification and Immunolocalization of Auxin in *Prunus dulcis* (Mill.) D. A. Webb Micrografts †

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Abstract: Almond (*Prunus dulcis* (Mill.) D. A. Webb) is a traditional culture in Portugal, which gained a renewed interest due to the installation of new orchards. Grafting remains the main method used for almond propagation. The successful establishment of a graft union between two parts (scion and rootstock) requires auxins, which are involved in wound response and vascular regeneration. This work aimed at the quantification and immunolocalization of indole-3-acetic acid (IAA) in almond micrografts before micrografting (T0) and 21 days after micrografting (T2). The results are a step forward to understand of how auxin is involved in graft compatibility in almond.

Keywords: almond; auxin; immunohistochemistry; indole-3-acetic acid; in vitro culture; micrografts

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1. Introduction

In recent years, almond production has increased due to the strong tendency of consumers towards plant-based products. One of the main almond propagation methods is grafting [1], a technique that joins two parts, scion and rootstock, to improve fruit quality, increase production, increase tolerance to biotic and abiotic stresses and/or improve edaphoclimatic adaptation [2]. Nevertheless, grafting is a challenging technique, and after wounding the plants, the scion is brought in contact with the rootstock, and through cell dedifferentiation and division, the *callus* is formed. Then, the full regeneration of functional vascular tissues establishes a connection between organs of different plants [3]. During grafting, hormonal signaling is involved in graft union formation, scion–rootstock communication and plant growth and development [4]. Among other plant growth regulators (PGRs), auxins are reported to be involved in the development of successful graft unions [5].

Since auxins are transported from stem apical regions to the roots, the plant vasculature transport dynamics are affected after cutting, during grafting, causing an accumulation of auxins in the scion and a depletion in the stock [3]. In *Arabidopsis thaliana*, it was shown that auxin accumulation in the grafted zone is followed by cell differentiation and vascular reconnection between scion and rootstock [6].

In spite of its practical importance and biological relevance, the mechanisms involved in scion–rootstock interactions are still poorly understood, but the role of auxins during grafting has been discussed [5,7,8].

In order to evaluate auxin role in grafting of *Prunus dulcis*, the quantification and immunolocalization of endogenous indole-3-acetic acid (IAA) in almond micrografts (homografts and heterografts) were performed. This study will contribute to increasing the knowledge of auxin's influence on micrografting in *Prunus* spp.

2. Materials and Methods

2.1. Plant Materials

Canhota, a Portuguese traditional variety, and bitter almond plants were established in vitro from seedlings and micropropagated in MS (Murashige and Skoog, 1962) medium supplemented with 6-Benzylaminopurine (BAP, 0.1 mg/L), 3% (*w*/*v*) sucrose and 0.7% (*w*/*v*) agar. The pH was adjusted to 5.7 and autoclaved for 20 min at 121 °C. Cultures were kept in a growth chamber at 25 ± 1 °C and 16 h light/8 h dark photoperiod.

2.2. Micrografts

Micrografts were established using bitter almond homografts and bitter almond (rootstock) × Canhota (scion) heterografts. Under sterile conditions, apical shoot segments of approximately 1 cm were used as scion and basal shoot segments, with the same length used for rootstocks. A 'v' cut was made, and the scion was inserted in the stock. For each combination, 10 micrografts were placed in plastic containers (125 × 65 × 80 mm Combiness box, with white filters) containing MS medium supplemented with 3% (*w*/*v*) sucrose and 0.7% (*w*/*v*) agar and kept in the same conditions.

2.3. IAA Quantification

The IAA content was accessed using the colorimetric method described by Anthony and Street (1969). The plant material (3–60.5 mg tissue fresh weight (FW)) was ground in a mortar with liquid nitrogen. A volume of Na-phosphate buffer 0.01 M (pH 7.0) 3-fold greater than the FW was added, and after centrifugation (17,000× *g* rpm; 12 min), the supernatant was recovered, and Na-phosphate buffer 0.01 M (pH 7.0) was added until the final volume of 1 mL. To the diluted sample, 2 mL of 100% (*w*/*v*) trichloroacetic acid (TCA) and 2 mL of Ehrlich's reagent (Sigma-Aldrich, St. Louis, MO, USA) were added in order. A blank solution of Na-phosphate buffer 0.01 M was prepared simultaneously. Reaction occurred for 20 min in the dark, and the absorbance was measured at 530 nm in a Jenway 7305 spectrometer. For each segment, 3 replicates were used, and the results obtained in μg of IAA per mg of fresh tissue.

2.4. IAA Immunolocalization

Samples were fixated by complete immersion in a solution of ice-cold 4% (*w*/*v*) paraformaldehyde overnight on an orbital shaker under gentle shaking. For sample dehydration, the fixative was replaced by ice-cold filtered 10% (*w*/*v*) sucrose solution in 1× phosphate-buffered saline (PBS), pH 7.4, applied o/n at 4 °C, which was then replaced by a new ice-cold filtered 20% (*w*/*v*) sucrose solution in 1× PBS, pH 7.4, for a second overnight period at 4 °C. In every solution replacement, vacuum was applied for 20 min, and samples were kept in an orbital shaker under gentle shaking. Samples were embedded in Optimum Cutting Temperature compound (O.C.T.) (Tissue-Tek TM, Sakura) and kept at −80 °C before cryosectioning. Sections of 10 μm were cut in a cryostat (CM3050 S Leica Microsystems, Nussloch, Germany), were collected on coated slides and permeabilized with 2% (*w*/*v*) driselase in 8.5% (*w*/*v*) D-mannitol solution at 37 °C for 30 min before treatment. Samples were pre-treated with blocking solution (10% bovine serum albumin (BSA) in 1× PBS for 30 min), washed with 1× PBS for 5 min and incubated with anti-IAA primary antibody (Ref.: AS09 421 AGRISERA, Vännäs, Sweden) in a 1:200 dilution in 1% BSA in 1× PBS overnight in a dark moistened chamber. The secondary antibody, Alexa Fluor® 633 goat anti-rabbit (Molecular Probes, Göttingen, Germany), was diluted 1:200 in 1% BSA in 1× PBS, and sections were incubated for 1 h in a dark moistened chamber. Samples were washed twice with 1× PBS for 2 min and slides assembled with Dako

Fluorescence Mounting Medium (Agilent, Santa Clara, CA, USA). Negative controls were obtained by omitting the incubation step with the primary antibody. Images were acquired in a Zeiss Axio Observer.Z1 inverted microscope (equipped with a AxioCam HRm and Zen Blue 2012 software (all from Carl Zeiss, Jena, Germany)) using an A-Plan 2.5×/0.06 differential interface contrast (DIC) and EC Plan-Neofluar 10×/0.3 Ph1 objectives and R 631/633 nm laser (Colibri 7 LED light source). Images were processed with Fiji Software (version 1.53c).

IAA quantification and immunolocalization assays were carried out before micrografting (T0) and 21 days after micrografting (T2). T0 samples correspond to cut but ungrafted scions and rootstocks. Micrografts collected at T2 were segmented in scion, union and rootstock.

2.5. Statistical Analysis

Statistical analysis was performed using Graph Pad Prism (Version 8.4.3 (686), San Diego, CA, USA). Tukey's multiple comparison test at $p \leq 0.05$ was used to analyze IAA content in ungrafted scions and rootstocks at T0 and in the micrograft segments at T2.

3. Results and Discussion

3.1. IAA Quantification

The established micrograft combinations (Figure 1) resulted in different micrografting success rates after 21 days, with 60% and 90% in bitter almond × bitter almond and bitter almond × Canhota, respectively.

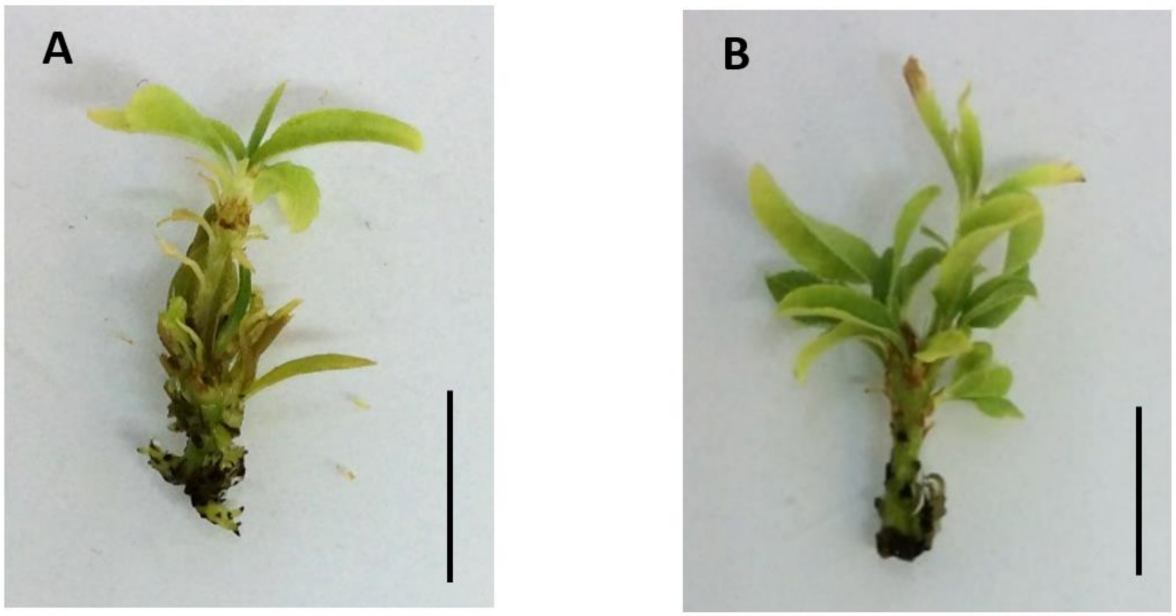

Figure 1. Micrografts 21 days after micrografting: (**A**) Bitter almond × bitter almond and (**B**) bitter almond × Canhota. Scale bar—1 cm.

The IAA quantification at T0 showed significant differences ($p \leq 0.05$) between scions (Canhota and bitter almond) and between the Canhota scion and bitter almond rootstock (Figure 2). The Canhota scion presented an IAA content of 1.292 ± 0.448 µg IAA/ mg FW, lower than the 5.505 ± 1.179 µg IAA/ mg FW observed in bitter almond scion and the 4.107 ± 1.253 µg IAA/ mg FW in bitter almond rootstock.

Chen et al. (2017) analyzed the IAA concentrations of compatible and incompatible combinations of grafted *Litchi chinensis* and revealed an initial decrease in IAA concentrations in compatible grafts. In addition to these observations, here, the micrograft combinations established with Canhota, which revealed significantly lower IAA initial content, presented higher micrografting success. Since both combinations were established with the

same rootstock, the difference in the micrografting success rates could be linked to the IAA initial levels in scions and rootstock, and initial lower levels of IAA in the scion could lead to a higher micrografting success rate.

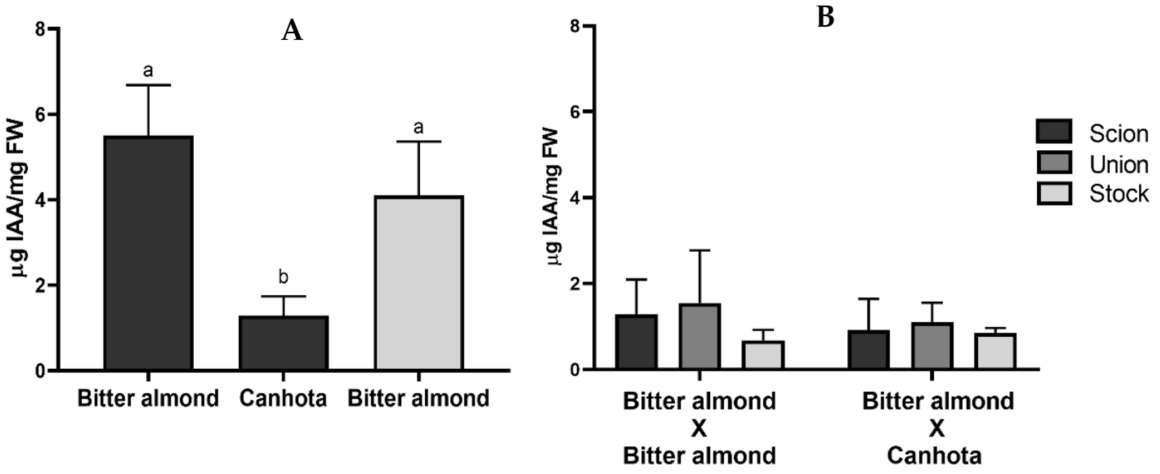

Figure 2. IAA quantification (**A**) in bitter almond and Canhota varieties of ungrafted scions and rootstocks (T0) and (**B**) in bitter almond homografts and Canhota grafted onto bitter almond segments at 21 days after grafting (T2) using Ehrlich reaction. IAA is presented in µg of IAA per mg of tissue FW. Error bars correspond to SD (n = 3). Values indicated with different letters were statistically different at $p \leq 0.05$ using Tukey's multiple comparison test.

At T2, the IAA content of micrograft segments (scion, union and rootstock) (Figure 2) was not statistically different ($p > 0.05$) in both bitter almond x bitter almond and bitter almond × Canhota. However, IAA was present in all micrograft segments, including the graft union, where an IAA content of 1.547 ± 1.225 µg IAA/mg FW was observed in homografts and 1.103 ± 0.451 µg IAA/ mg FW in heterografts. Auxin has been described in wound response and vascular formation; thus, it is unsurprising that auxins are involved in micrografting [7]. The presence of IAA at the graft union could be associated with the described role of IAA in the graft compatibility [9], that was proved by some authors by the exogenous application of IAA, which improved grafting success [8]. In *A. thaliana*, the accumulation of auxin at the graft union was followed by cell differentiation and vascular reconnection, crucial steps for the success of grafting [6], and even though the presence of IAA at the graft union was detected here, the following events need further investigation.

3.2. IAA Immunolocalization

At T0, an IAA signal was detected in Canhota scion and in bitter almond rootstock (Figure 3). The observation of more intense labelled IAA in Canhota shoot apex is in accordance to the reported auxin synthesis in young growing regions of plants, in the shoot apex and in young leaves [10]. This analysis was performed immediately after cutting the segments, and the presence of fluorescence in the bitter almond rootstock might indicate that auxin transport, from the main synthesis organs to the roots, is not yet affected.

At T2, an IAA signal was detected in the scion segment and in the graft union, apparently in the scion part (Figure 4). In the rootstock, auxin is apparently absent close to the graft union, and more intensively labelled closer to the base. The changes in auxin transport comparatively to T0 may have resulted from the vasculature cut, but further studies are needed. This asymmetric accumulation of auxin, also described in *A. thaliana*, is probably required for an efficient regeneration and reconnection of vascular tissues during grafting [3].

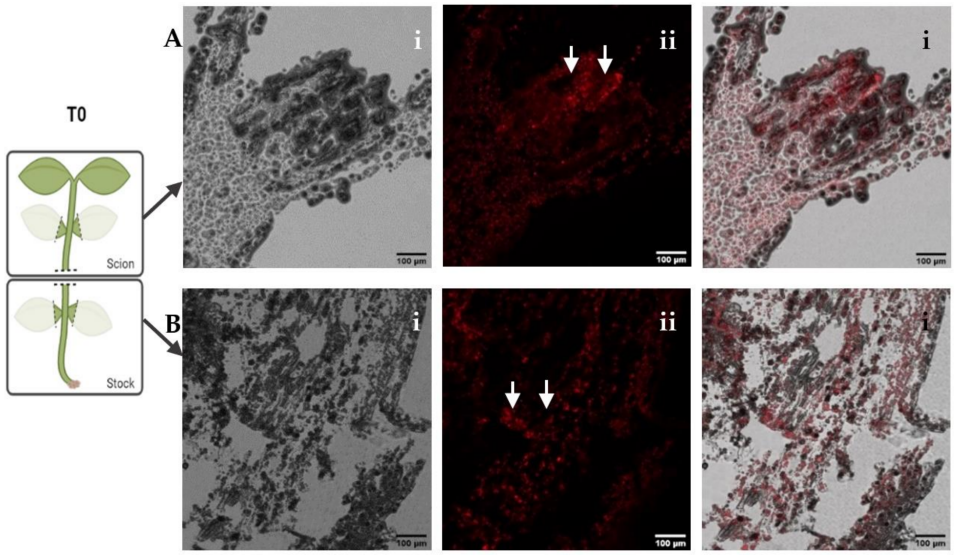

Figure 3. Immunolocalization of IAA in 4% (*v*/*v*) paraformaldehyde fixed histological sections (10 µm) of Canhota scion (**A**) and bitter almond stock (**B**) at T0: (i) Sections observed under transmission light (right column) were used for histological control; (ii) Sections stained with the anti-IAA antibody and Alexa Fluor® 633 (center column); (iii) Merged images. Arrows indicate the places where IAA has been more intensively labelled. Scale bar—100 µm.

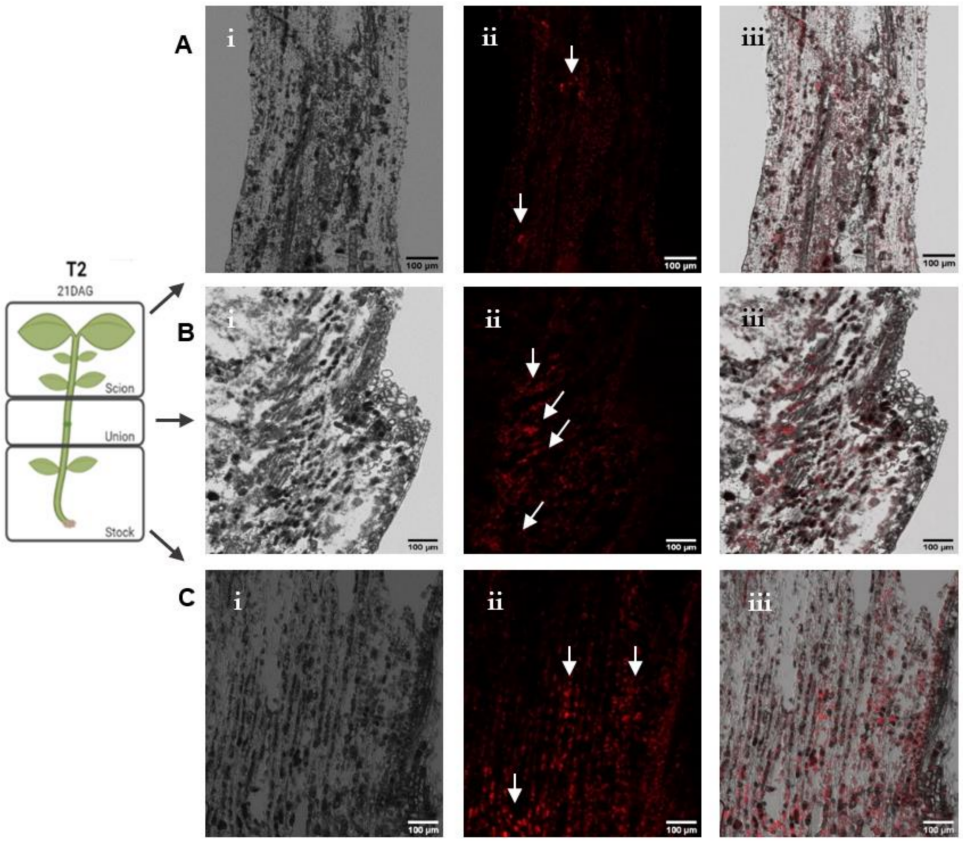

Figure 4. Immunolocalization of IAA in 4% (*v*/*v*) paraformaldehyde fixed histological sections (10 µm) of bitter almond × bitter almond micrografts scion (**A**), graft union (**B**) and stock (**C**) at T2: (i) Sections observed under transmission light (right column) were used for histological control; (ii) Sections stained with the anti-IAA antibody and Alexa Fluor® 633 (center column); (iii) Merged images. Arrows indicate the places where IAA has been more intensively labelled Scale bar—100 µm.

4. Conclusions

Auxin quantification in almond micrografts revealed a possible influence of scion IAA initial levels on micrografting success and the presence of IAA at the graft union 21 days after micrografting. In ungrafted scions and rootstocks, the IAA transport dynamics do not appear to be affected by the cut. However, 21 days after micrografting, IAA was also present at the graft union, which could be the first signs for the successful establishment of compatible micrografts. These results are a step forward in the investigation of auxin's role in the grafting of *P. dulcis* and are a base for future studies on the molecular communication between the scion and rootstock in almond grafts.

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